Title of the Invention

EXPRESSION OF LYSOSOMAL HYDROLASE IN CELLS EXPRESSING PRO-N-ACETYLGLUCOSAMINE-1-PHOSPHODIESTER ALPHA-N-ACETYL GLUCOSIMANIDASE

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention provides methods of producing a pro-N-acetylglucosamine-1-phosphodiester α N-acetyl glucosimanidase (phosphodiester α-GlcNAcase), in mammalian cells deficient in the furin proteolytic enzyme and methods of making lysosomal hydrolases having oligosaccharides modified with N-acetylglucosamine-1-phosphate.

Discussion of the Background

Lysosomes are organelles in eukaryotic cells that function in the degradation of macromolecules into component parts that can be reused in biosynthetic pathways or discharged by the cell as waste. Normally, these macromolecules are broken down by enzymes known as lysosomal enzymes or lysosomal hydrolases. However, when a lysosomal enzyme is not present in the lysosome or does not function properly, the enzymes specific macromolecular substrate accumulates in the lysosome as "storage material" causing a variety of diseases, collectively known as lysosomal storage diseases.

Lysosomal storage diseases can cause chronic illness and death in hundreds of individuals each year. There are approximately 50 known lysosomal storage diseases, e.g., Pompe Disease, Hurler Syndrome, Fabry Disease, Maroteaux-Lamy Syndrome

(mucopolysaccharidosis VI), Morquio Syndrome (mucopolysaccharidosis IV), Hunter Syndrome (mucopolysaccharidosis II), Farber Disease, Acid Lipase Deficiency, Krabbe Disease, and Sly Syndrome (mucopolysaccharidosis VII). In each of these diseases, lysosomes are unable to degrade a specific compound or group of compounds because the enzyme that catalyzes a specific degradation reaction is missing from the lysosome, is present in low concentrations in the lysosome, or is present at sufficient concentrations in the lysosome but is not functioning properly.

Lysosomal Storage diseases have been studied extensively and the enzymes (or lack thereof) responsible for particular diseases have been identified (Scriver, Beaudet, Sly, and Vale, eds., The Metabolic Basis of Inherited Disease, 6th Edition, 1989, Lysosomal Enzymes, Part 11, Chapters 61-72, pp. 1565-1839). Within each disease, the severity and the age at which the disease presents may be a function of the amount of residual lysosomal enzyme that exists in the patient.

The lysosomal targeting pathways have been studied extensively and the process by which lysosomal enzymes are synthesized and transported to the lysosome has been well described. Kornfeld, S. (1986). "Trafficking of lysosomal enzymes in normal and disease states." *Journal of Clinical Investigation* 77: 1-6 and Kornfeld, S. (1990). "Lysosomal enzyme targeting." *Biochem. Soc. Trans.* 18: 367-374. Generally, lysosomal enzymes are synthesized by membrane-bound polysomes in the rough endoplastic reticulum ("RER") along with secretory glycoproteins. In the RER, lysosomal enzymes acquire N-linked oligosaccharides by the en-bloc transfer of a preformed oligosaccharide from dolichol phosphate containing 2 N-acetylglucosamine, 9-mannose and 3-glucose. Glycosylated lysosomal enzymes are then transported to the Golgi apparatus along with secretory proteins. In the cis-Golgi or intermediate compartment lysosomal enzymes are specifically and uniquely modified by the transfer of GlcNAc-phosphate to specific mannoses. In a second step, the GlcNAc is removed thereby exposing the mannose 6-phosphate ("M6P") targeting determinant. The

lysosomal enzymes with the exposed M6P binds to M6P receptors in the trans-Golgi and is transported to the endosome and then to the lysosome. In the lysosome, the phosphates are rapidly removed by lysosomal phosphatases and the mannoses are removed by lysosomal mannosidases (Einstein, R. and Gabel, C.A. (1991). "Cell- and ligand-specific deposphorylation of acid hydrolases: evidence that the mannose 6-phosphate is controlled by compartmentalization." *Journal of Cell Biology* 112: 81-94).

The synthesis of lysosomal enzymes having exposed M6P is catalyzed by two different enzymes, both of which are essential if the synthesis is to occur. The first enzyme is UDP-N-acetylglucosamine: lysosomal enzyme N-Acetylglucosamine-1-phosphotransferase ("GlcNAc-phosphotransferase"). GlcNAc-phosphotransferase catalyzes the transfer of N-acetylglucosamine-1-phosphate from UDP-GlcNAc to the 6 position of 1,2-linked mannoses on the lysosomal enzyme. The recognition and addition of N-acetylgluocosamine-1-phosphate to lysosomal hydrolases by GlcNAc-phosphotransferase is the critical and determining step in lysosomal targeting. The second step is catalyzed by N-acetylglucosamine-1-phosphodiester -N-Acetylglucosaminidase ("phosphodiester α-GlcNAcase"). Phosphodiester α-GlcNAcase catalyzes the removal of N-Acetylglucosamine from the GlcNAc-phosphate modified lysosomal enzyme to generate a terminal M6P on the lysosomal enzyme.

The present inventors have discovered that the phosphodiester α-GlcNAcase comprises a pro-peptide sequence between the signal sequence and the sequence of the active component of the protein sequence. This pro-peptide sequence is proteolytically cleaved to yield a mature active form of phosphodiester α-GlcNAcase. The activity of uncleaved phosphodiester α-GlcNAcase, i.e., containing the pro-peptide sequence was significantly lower than the activity of the phosphodiester α-GlcNAcase when the pro-peptide sequence was cleaved. The inventors have revealed that the pro-peptide sequence contains a recognition site for the site-specific protease Furin and that Furin mediates cleavage of phosphodiester α-GlcNAcase to it's mature form.

SUMMARY OF THE INVENTION

Based on this finding, the invention provides processes of making lysosomal hydrolase in cells which are deficient in Furin and thus have the uncleaved form of phosphodiester α-GlcNAcase. By making the lysosomal hydrolases in these cells, the lysosomal hydrolase is modified with an N-acetylglucosamine-1-phosphate moiety and is not removed, or removed at a low efficiency. After expression and recovery of the lysosomal hydrolase from these Furin deficient cells, the lysosomal hydrolase can be treated with an active form of phosphodiester α-GlcNAcase thereby removing the N-acetylglucosamine moiety to yield a highly phosphorylated lysosomal enzyme, which can be used in enzyme replacement therapies to treat patients suffering from lysosomal storage diseases.

Thus, the method facilitates a simple and reliable method of producing lysosomal hydrolases with the appropriate phospho-modifications thereby reducing the steps necessary to produce a lysosomal hydrolase for therapeutic or experimental use. Additional advantages include that the N-acetylglucosamine-1-phosphate modified oligosaccharides will not bind to trans-Golgi mannose 6-phosphate receptors resulting in secretion of a greater proportion synthesized lysosomal enzyme thereby improving the yield. Additionally, because less lysosomal enzyme is trafficked to the lysosome there should be less processing to mature forms facilitating the preparation of pure precursor lysosomal enzyme preparations. Oligosaccharides not modified by N-acetylglucosamine-1-phosphate should be processed to complex-type oligosaccharides reducing the number

of mannose contained in the lysosomal enzyme thereby reducing affinity for mannose receptors.

Accordingly, an object of the present invention is to provide methods of producing lysosomal hydrolases having an Oligosaccharide modified with N-acetylglucosamine-1-phosphateby expressing a nucleotide sequence encoding the lysosomal hydrolase in a mammalian cell that is deficient in the protease Furin.

Another object of the present invention is methods for producing a phosphodiester α-GlcNAcase having its pro-peptide intact by culturing cells or selecting cells that are furin deficient, where the selection is preferably conducted using *Pseudomonas* exotoxin A.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Western Blot of phosphodiester α-GlcNAcase in the presence or absence of Furin.

Figure 2. Phosphodiester α-GlcNAcase Activity in the presence or absence of Furin.

Figure 3. Mannose-6-phosphate binding capacity of β-glucuronidase (β-Gluc) and N-acetyl-β-D-glucosaminidase (NAcGluc). (A) Conditioned media after 24 hours of LoVo culture. (B) Conditioned media after Alkaline Phosphatase treatment. (C) Conditioned media after rh-UCE treatment. (D) Conditioned media after rh-UCE treatment, followed by Alkaline Phosphatase digestion.

DETAILED DESCRIPTION OF THE INVENTION

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art of molecular biology. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described herein. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In addition, the materials, methods, and examples are illustrative only and are not intended to be limiting.

Reference is made to standard textbooks of molecular biology that contain definitions and methods and means for carrying out basic techniques, encompassed by the present invention. See, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, Third Edition, Cold Spring Harbor Laboratory Press, New York (2001), Current Protocols in Molecular Biology, Ausebel et al (eds.), John Wiley & Sons, New York (2001) and the various references cited therein.

"Isolated" means separated out of its natural environment.

"Polynucleotide" in general relates to polyribonucleotides and polydeoxyribonucleotides, it being possible for these to be non-modified RNA or DNA or modified RNA or DNA.

The term "nucleotide sequence" as used herein means a polynucleotide molecule in the form of a separate fragment or as a component of a larger nucleic acid construct that has been derived from DNA or RNA isolated at least once in substantially pure form *(i.e.,* free of contaminating endogenous materials) and in a quantity or concentration

enabling identification, manipulation, and recovery of its component nucleotide sequences by standard biochemical methods. Such sequences are preferably provided in the form of an open reading frame uninterrupted by internal non-translated sequences, or introns that are typically present in eukaryotic genes. Sequences of non-translated DNA may be present 5' or 3' from an open reading frame where the same do not interfere with manipulation or expression of the coding region.

The term "nucleic acid molecule" as used herein means RNA or DNA, including cDNA, single or double stranded, and linear or covalently closed molecules. A nucleic acid molecule may also be genomic DNA corresponding to the entire gene or a substantial portion therefor to fragments and derivatives thereof. The nucleotide sequence may correspond to the naturally occurring nucleotide sequence or may contain single or multiple nucleotide substitutions, deletions and/or additions including fragments thereof. All such variations in the nucleic acid molecule retain the ability to encode a biologically active enzyme when expressed in the appropriate host or an enzymatically active fragment thereof. The nucleic acid molecule of the present invention may comprise solely the nucleotide sequence encoding an enzyme or may be part of a larger nucleic acid molecule that extends to the gene for the enzyme. The non-enzyme encoding sequences in a larger nucleic acid molecule may include vector, promoter, terminator, enhancer, replication, signal sequences, or non-coding regions of the gene.

Transcriptional and translational control sequences for mammalian host cell expression vectors may be excised from viral genomes. Commonly used promoter sequences and enhancer sequences are derived from Polyoma virus, Adenovirus 2, Simian Virus 40 (SV40), and human cytomegalovirus. DNA sequences derived from the

SV40 viral genome may be used to provide other genetic elements for expression of a structural gene sequence in a mammalian host cell, *e.g.,* SV40 origin, early and late promoter, enhancer, splice, and polyadenylation sites. Viral early and late promoters are particularly useful because both are easily obtained from a viral genome as a fragment which may also contain a viral origin of replication. Other control or regulatory sequences can be employed as is known in the art. Exemplary expression vectors for use in mammalian host cells are well known in the art.

Methods of introducing, transducting or transfecting mammalian cells are well within the knowledge of the skilled artisan. Examples of such methods include calcium phosphate-mediated, liposome-mediated, Dextran-mediated, and electroporation. These and other methods are described in, for example, Sambrook et al (2001) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, NY and Current Protocols in Molecular Biology (2001) and Ausebel et al (eds.), John Wiley and Sons, Inc, New York.

According to the present invention, the glycoproteins may be produced by the recombinant expression systems described above. Thc method comprises culturing a host cell transformed with an expression vector comprising a DNA sequence that encodes the glycoprotein under conditions sufficient to promote expression of the glycoprotein.

"Polypeptides" are understood as meaning peptides or proteins which comprise two or more amino acids bonded via peptide bonds.

"Glycoprotein" as used herein means proteins that are endogenously modified to carry one or more carbohydrate moieties on the protein. Within the context of the present invention, lysosomal hydrolase glycoproteins are preferred. Examples of lysosomal

hydrolases include α-glucosidase, α -L-iduronidase, α -galactosidase A, arylsulfatase , N-acetylgalactosamine-6-sulfatase or β -galactosidase, iduronate 2-sulfatase, ceramidase, galactocerebrosidase, β-glucuronidase, Heparan N-sulfatase, N-Acetyl-α-glucosaminidase, Acetyl CoA-α-glucosaminide N-acetyl transferase, N-acetyl-glucosamine-6 sulfatase, Galactose 6-sulfatase, Arylsulfatase A, B, and C, Arylsulfatase A Cerebroside, Ganglioside, Acid β-galactosidase G_{M1} Galglioside, Acid β-galactosidase, Hexosaminidase A, Hexosaminidase B, α-fucosidase, α-N-Acetyl galactosaminidase, Glycoprotein Neuraminidase, Aspartylglucosamine amidase, Acid Lipase, Acid Ceramidase, Lysosomal Sphingomyelinase and other Sphingomyelinases.

The term "biologically active" as used herein means an enzyme or protein having structural, regulatory, or biochemical functions of a naturally occurring molecule.

"Complex carbohydrates" as used herein means contains monosaccharide other than GlnAc and mannose (Kornfeld, R and Kornfeld, S. (1985) Ann Rev Biochem 54:631-664).

In the present invention any mammalian cell can be utilized, primary or established. Preferably, the mammalian cell is an established cell line that proliferates in culture and is amenable to selection as described herein. Examples of such cells include HeLa, 293T, Vero, NIH 3T3, Chinese Hamster Ovary, and NS0.

Mammalian cells can be cultured in dishes, plates, and flasks in the appropriate medium in accordance with standard cell culture protocols (Sambrook et al (2001) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, NY and Current Protocols in Molecular Biology (2001) and Ausebel et al (eds.), John Wiley and Sons, Inc, New York). As recognized by the skilled artisan the type of vessel and

specific culture conditions will vary depending on the specific cell type, whether the cell is typically cultured in suspension, adherent or in a co-culture with one or more cells.

The GlcNAc-phosphotrasferase is composed of six subunits: 2 α subunits, 2 β-subunits and 2 γ subunits. The amino acid sequence of the α subunit is shown in SEQ ID NO:4 (amino acids 1-928), the human β subunit is shown in SEQ ID NO:5 (amino acids 1-328), and the human γ subunit is shown in SEQ ID NO:7 (amino acids 25-305, signal sequence is in amino acids 1-24).

In another embodiment, the GlcNAc-phosphotransferase is recombinant GlcNAc-phosphotransferase, which has been engineered to remove the membrane binding domain from the polyprotein containing the α/β subunits and the endogenous proteolytic cleavage site is replaced with a non-endogenous site-specific proteolytic cleavage site such as Furin, Factor Xa , Enterokinase, and Genease. After preparing the α/β subunits they can be combined with an isolated γ-subunit to yield a GlcNAc-phosphotransferase enzyme.

The soluble GlcNAc-phosphotransferase protein or polypeptide include the sequences exemplified in this application as well as those which have substantial identity to SEQ ID NO:2.

The partial rat and Drosphila melanogaster α/β GlcNAc-phosphotransferase amino acid sequences are shown in SEQ ID NO: 14 and 16, respectively.

Preferably, the GlcNAc-phosphotransferase polypeptides are those which are at least 70%, preferably at least 80% and more preferably at least 90% to 95% identical to the GlcNAc-phosphotransferase amino acid sequences described herein.

Polynucleotides which encode the α and β subunits of GlcNAc-phosphotransferase or soluble GlcNAc-phosphotransferase mean the sequences exemplified in this application as well as those which have substantial identity to those sequences and which encode an enzyme having the activity of the α and β subunits of GlcNAc-phosphotransferase. Preferably, such polynucleotides are those which hybridize under stringent conditions and are at least 70%, preferably at least 80% and more preferably at least 90% to 95% identical to those sequences

The nucleotide sequence for the human α/β subunit precursor cDNA is shown in SEQ ID NO:3 (nucleotides 165-3932), the nucleotide sequence of the α subunit is in nucleotides 165-2948 of SEQ ID NO:3, the nucleotide sequence of the β subunit is shown in nucleotides 2949-3932 of SEQ ID NO:3, and the nucleotide sequence of the γ subunit is shown in SEQ ID NO:6 (nucleotides 24-95). The soluble GlcNAc-phosphotransferase nucleotide sequence is shown in SEQ ID NO:1. The partial rat and Drosphila melanogaster α/β GlcNAc-phosphotransferase nucleotide sequences are shown in SEQ ID NO: 13 and 15, respectively.

Polynucleotides which encode phosphodiester α -GlcNAcase as used herein is understood to mean the sequences exemplified in this application as well as those which have substantial identity to SEQ ID NO:19 (murine) or SEQ ID NO:17 (human) and which encode an enzyme having the activity of phosphodiester α -GlcNAcase. Preferably, such polynucleotides are those which hybridize under stringent conditions and are at least 70%, preferably at least 80% and more preferably at least 90% to 95% identical to SEQ ID NOS:17 and/or 19.

The phosphodiester α -GlcNAcase protein or polypeptide as used herein is understood to mean the sequences exemplified in this application as well as those which have substantial identity to SEQ ID NO:20 (murine) or SEQ ID NO:18 (human). Preferably, such polypeptides are those which are at least 70%, preferably at least 80% and more preferably at least 90% to 95% identical to SEQ ID NOS:18 and/or 20.

Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C. for short probes (e.g., 10 to 50 nucleotides) and at least about 60° C. for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37° C., and a wash in 1X to 2X SSC (20X SSC=3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1 M NaCl, 1% SDS at 37°C., and a wash in 0.5X to 1X SSC at 55 to 60oC. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C., and a wash in 0.1X SSC at 60 to 65°C.

Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA--DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl, Anal. Biochem., 138:267-284 (1984): T_m =81.5°C.+16.6 (log M)+0.41 (%GC)-0.61 (% form)-500/L; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide

in the hybridization solution, and L is the length of the hybrid in base pairs. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. T_m is reduced by about 1°C. for each 1% of mismatching; thus, T_m, hybridization and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with approximately 90% identity are sought, the T_m can be decreased 10°C. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4°C. lower than the thermal melting point (T_m); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10°C. lower than the thermal melting point (T_m); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20°C. lower than the thermal melting point (T_m). Using the equation, hybridization and wash compositions, and desired T_m, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than 45°C. (aqueous solution) or 32°C. (formamide solution) it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen, Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes, Part I, Chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays", Elsevier, New York (1993); and Current Protocols in Molecular Biology, Chapter 2, Ausubel, et al., Eds., Greene Publishing and Wiley-Interscience, New York (1995).

Homology, sequence similarity or sequence identity of nucleotide or amino acid sequences may be determined conventionally by using known software or computer programs such as the BestFit or Gap pairwise comparison programs (GCG Wisconsin Package, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin 53711). BestFit uses the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2: 482-489 (1981), to find the best segment of identity or similarity between two sequences. Gap performs global alignments: all of one sequence with all of another similar sequence using the method of Needleman and Wunsch, J. Mol. Biol. 48:443-453 (1970). When using a sequence alignment program such as BestFit, to determine the degree of sequence homology, similarity or identity, the default setting may be used, or an appropriate scoring matrix may be selected to optimize identity, similarity or homology scores. Similarly, when using a program such as BestFit to determine sequence identity, similarity or homology between two different amino acid sequences, the default settings may be used, or an appropriate scoring matrix, such as blosum45 or blosum80, may be selected to optimize identity, similarity or homology scores.

The furin deficient cells that are known and available to the skilled artisan may be employed, including but not limited to FD11 cells (Gordon et al (1997) Infection and Immunity 65(8):3370-3375), and those mutant cells described in Moehring and Moehring (1983) Infection and Immunity 41(3):998-1009.

Alternatively, a furin deficient cell may be obtained by exposing cultured cells to mutagenesis treatment, e.g., irradiation, ethidium bromide, bromidated uridine (BrdU) and others, preferably chemical mutagenesis, and more preferred ethyl methane sulfonate mutagenesis, recovering the cells which survive the treatment and selecting for those

cells which are found to be resistant to the toxicity of *Pseudomonas* exodotoxin A (see Moehring and Moehring (1983) Infection and Immunity 41(3):998-1009).

The amount of *Pseudomonas* exodotoxin A can be used as described *supra*, or can be empirically determined for each individual cell type by titrating various concentration of *Pseudomonas* exodotoxin A on the cells and observing the concentration of *Pseudomonas* exodotoxin A, which does not result in the killing of all the cells. A preferred range includes 0.5 to 2.0 μg/ml, including 0.75, 1.0, 1.25, 1.5, 1.75, and all values therebetween.

The phrase "highly phosphorylated lysosomal hydrolase" as used herein refers to lysosomal hydrolases which contains more bis-phosphorylated oligosaccharides compared to known naturally occurring or recombinant lysosomal hydrolases. Preferably, the lysosomal hydrolases contains at least 5% bis-phosphorylated oligosaccharides compared to lysosomal hydrolases not treated with the GlcNAc-phosphotransferase described herein. More preferably, the "highly phosphorylated lysosomal hydrolases" has at least 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%,14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%,23%, 24%, 25%, 26%, 27%, 28%, 29%,30%, 40%,45%, 50%, 60%, 70%, 80%, 85%, 90%, 95%, 100% bis-phosphorylated oligosaccharides and all values and ranges there between. This highly phosphorylated lysosomal hydrolases have a higher affinity for the M6P receptor and are therefore more efficiently taken into the cell by plasma membrane receptors.

To determine the extent to which the lysosomal hydrolase is phosphorylated, the lysosomal hydrlase pre- and post-phosphorylation treatment can be assayed by binding to

Mannose-6-phosphate as described herein and in Hoflack et al (1985) *Biochem* 260:12008-120014.

When the cells are also furin deficient are employed the resultant lysosomal hydrolases containing the N-acetylglucosamine-1-phosphate is obtained due to the significantly lower phosphodiester-α-GlcNAcase activity. The purified lysosomal hydrolases having a Oligosaccharide modified with N-acetylglucosamine-1-phosphateis then treated *in vitro* with with phosphodiester α GlcNAcase to remove the N-acetylglucosamine moiety.

In another embodiment of the invention, the cells found to be furin deficient may also be subsequently or previously selected for lectin resitance, preferably ricin resistance as described in Applicants co-pending U.S. applications: "METHOD OF PRODUCING GLYCOPROTEINS HAVING REDUCED COMPLEX CARBOHYDRATES IN MAMMALIAN CELLS" or METHODS OF PRODUCING HIGH MANNOSE GLYCOPROTEINS IN COMPLEX CARBOHYDRATE DEFICIENT CELLS", the contents of which are incorporated herein by reference.

Any lysosomal enzyme that uses the M6P transport system can be treated according to the method of the present invention. Examples include α-glucosidase (Pompe Disease), α–L-iduronidase (Hurler Syndrome), β-galactosidase A (Fabry Disease), arylsulfatase (Maroteaux-Lamy Syndrome), N-acetylgalactosamine-6-sulfatase or -galactosidase (Morquio Syndrome), iduronate 2-sulfatase (Hunter Syndrome), ceramidase (Farber Disease), galactocerebrosidase (Krabbe Disease), β -glucuronidase (Sly Syndrome), Heparan N-sulfatase (Sanfilippo A), N-Acetyl-α-glucosaminidase (Sanfilippo B), Acetyl CoA-α-glucosaminide N-acetyl transferase, N-acetyl-

glucosamine-6 sulfatase (Sanfilippo D), Galactose 6-sulfatase (Morquio A), Arylsulfatase A, B, and C (Multiple Sulfatase Deficiency), Arylsulfatase A Cerebroside (Metachromatic Leukodystrophy), Ganglioside (Mucolipidosis IV), Acid β-galactosidase G_{M1} Galglioside (G_{M1} Gangliosidosis), Acid β-galactosidase (Galactosialidosis), Hexosaminidase A (Tay-Sachs and Variants), Hexosaminidase B (Sandhoff), α-fucosidase (Fucsidosis), α-N-Acetyl galactosaminidase (Schindler Disease), Glycoprotein Neuraminidase (Sialidosis), Aspartylglucosamine amidase (Aspartylglucosaminuria), Acid Lipase (Wolman Disease), Acid Ceramidase (Farber Lipogranulomatosis), Lysosomal Sphingomyelinase and other Sphingomyelinase (Nieman-Pick).

Methods for treating any particular lysosomal hydrolase with the GlcNAc-phosphotransferase and phosphodiester α-GlcNAcase are within the knowledge of the skilled artisan. Generally, the lysosomal hydrolase at a concentration of about 10 mg/ml and phosphodiester α-GlcNAcase at a concentration of about 1000 units/mL and the system is allowed to incubate for 2 hours at 37°C at a pH of about 6-7 and any stabilizers or coenzymes required to facilitate the reaction. The modified lysosomal enzyme having highly phosphorylated oligosaccharides is then recovered by conventional means.

The phosphorylated lysosomal hydrolase can be administered to a patient suffering from the lysosomal storage disorder to replace the deficient hydrolase as appropriate. Thus, the present invention also provides methods for the treatment of lysosomal storage diseases by administering an effective amount of the phosphorylated lysosomal hydrolase of the present invention to a patient diagnosed with the respective disease. As used herein, being diagnosed with a lysosomal storage disorder includes pre-

symptomatic phases of the disease and the various symptomatic identifiers associated with the disease. Typically, the pre-symptomatic patient will be diagnosed with the disease by means of a genetic analysis known to the skilled artisan.

While dosages may vary depending on the disease and the patient, phosphorylated hydrolase are generally administered to the patient in amounts of from about 0.1 to about 1000 milligrams per kg of patient per month, preferably from about 1 to about 500 milligrams per kg of patient per month. Amongst various patients the severity and the age at which the disease presents itself may be a function of the amount of residual hydrolase that exists in the patient. As such, the present method of treating lysosomal storage diseases includes providing the phosphorylated lysosomal hydrolase at any or all stages of disease progression.

The hydrolase may be administered by any convenient means, conventionally known to those of ordinary skill in the art. For example, the enzyme may be administered in the form of a pharmaceutical composition containing the enzyme and a pharmaceutically acceptable carrier or by means of a delivery system such as a liposome or a controlled release pharmaceutical composition. The term "pharmaceutically acceptable" refers to molecules and compositions that are physiologically tolerable and do not typically produce an allergic or similar unwanted reaction such as gastric upset or dizziness when administered. Preferably, "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopoeia or other generally recognized pharmacopoeia for use in animals, preferably humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile

liquids, such as saline solutions, dextrose solutions, glycerol solutions, water and oils emulsions such as those made with oils of petroleum, animal, vegetable, or synthetic origin (peanut oil, soybean oil, mineral oil, or sesame oil). Water, saline solutions, dextrose solutions, and glycerol solutions are preferably employed as carriers, particularly for injectable solutions.

The hydrolase or the composition may be administered by any standard technique compatible with enzymes or their compositions. For example, the enzyme or composition can be administered parenterally, transdermally, or transmucosally, *e.g.*, orally or nasally. Preferably, the hydrolase or composition is administered by intravenous injection.

As described above, the present invention also provides methods of obtaining or producing a phosphodiester α-GlcNAcase from cells deficient in the furin protease. This enzyme can be obtained or produced in the known furin deficient cell lines or in cell lines produced in accordance with the disclosure herein. After the phosphodiester α-GlcNAcase is collected from the cells, it may be stored or immediately cleaved *in vitro* with a preparation, preferably purified preparation, of the Furin protease. This cleaved phosphodiester α-GlcNAcase can then be used to remove the N-acetylglucosamine-1-phosphate from the lysosomal hydrolases as described herein.

The following Examples provide an illustration of embodiments of the invention and should not be construed to limit the scope of the invention, which is set forth in the appended claims. In the following Examples, all methods described are conventional unless otherwise specified.

EXAMPLES

Differential Specific Activity of CHO and Insect-Expressed Human UCE

CHO K-1 cells were transfected with plasmid pKB6 that encodes an epitope-tagged, soluble human phosphodiester α-GlcNAcase ("Uncovering Enzyme" or UCE). Similarly, insect cells were infected with a baculovirus that contained the epitope-tagged human UCE cDNA (performed by Protein Sciences, Inc.). The UCE-conditioned media from each expression system was affinity-purified via a HPC4 antibody column. The HPC4 eluates were concentrated via Centricons and assayed using the synthetic substrate [^{3}H]GlcNAc-*P*-Man-α-Me to determine UCE activity. The UCE protein concentration was measured by either absorbance at a wavelength of 280nm or with a protein quantitation kit e.g., Micro BCA Assay (Pierce-Endogen) and Advanced Protein Assay (Cytoskeleton). The purification of CHO- and insect-expressed UCE is summarized below.

Sample	Volume (ml)	Protein Conc. (mg/ml)	Total Protein (mg)	UCE Activity (Units/ml)	Total UCE Activity (Units)	Specific Activity (Units/mg)	% yield	Fold Purif.
CHO-Expressed UCE:								
Conditioned Media	1400	17.4	24360	8.7×10^3	12.2×10^6	501	100	1
HPC4 Eluate	1	44.6	44.6	11×10^6	11.1×10^6	**2.5×10^5**	91	499
Insect-Expressed UCE:								
Conditioned Media	100	15.2	1520	6×10^3	6×10^5	395	100	1
HPC4 Eluate	1	0.52	0.52	2.85×10^5	2.85×10^5	**5.5×10^5**	48	1392

Summary of Results: The CHO-expressed human UCE was efficiently purified via the HPC4 antibody column (91% yield). Approximately 10mg of UCE was recovered

per liter of conditioned CHO media. In contrast, the recovery of the insect-derived UCE was nearly half that of the CHO-derived UCE sample and recovered only 2.5mg UCE per liter of 96hr post infected insect media. Interestingly, the specific activity of the insect-derived human UCE was approximately 2-fold higher than the CHO-derived UCE. The major difference between the two UCE species is that the UCE plasmid construct in the insect expression system lacked the UCE pro-sequence.

Protein Sequence of CHO and Insect-Expressed Human UCE

The major difference between the two UCE species is that the UCE plasmid construct in the insect expression system lacked the UCE pro-sequence. Human UCE is a homotetramer and each monomer is synthesized as a pre-pro-UCE that is processed *in vivo* to generate the mature UCE monomer. The specific activity data show that these two UCE species are functionally distinct. To determine whether a difference is a different translation processing of CHO and insect expressed UCE the following experiments were conducted.

The N-terminal primary amino acid sequence of UCE (amino acids 1-55 of SEQ ID NO:18) is shown below, the signal peptide is indicated at the N-terminus, the Pro-peptide sequence is underlined and the N-terminal starting amino acids for the mature UCE are shown.

Primary amino acid sequence of human UCE:

N- MATSTGRWLLLRLALFGFLWEASGGLDSGASRDDDLLLPYPRARARLPR **DCTRVR...**

Signal Peptide — Pro-peptide — Mature UCE

CHO and insect-derived UCE samples were subjected to SDS-PAGE and then transferred to PVDF membrane. The membrane was stained with Ponceau S stain to visualize the protein bands. The insect and UCE bands were excised from the membrane and subjected to N-terminal sequencing. The results are present in the Table below:

N-terminal Sequencing of rh-UCE:

	CHO-derived UCE:				**Insect-derived UCE:**		
Cycle # Processed	Amino Acid	% Unprocessed	% Processed		Cycle #	Amino Acid	%
1	L, D	69	31		1	D	100
2	D	100	not detected		2	not detected	-
3	S, T	60	40		3	T	100
4	G	100	not detected		4	R	100
5	A, V	68	32		5	V	100
6	S	100	not detected		6	R	100
7	R	100	not detected		7	A	100
8	D, G	64	36		8	G	100
9	D, N	55	45		9	N	100
10	D	100	not detected		10	not detected	-

These results demonstrate that there are major structural differences between the CHO and insect-derived rh-UCE. The CHO-derived UCE is not processed efficiently, i.e., ~65% pro-UCE and 35% mature UCE. In contrast, the insect-derived UCE is 100% mature UCE. The insect UCE was expected to exist only as the mature form because the plasmid lacked a pro-sequence. The data indicate that the majority of the CHO-derived UCE must have either escaped the processing enzyme(s) that converts pro-UCE to the mature UCE or that the processing enzyme(s) responsible for this cleavage is defective in this CHO cell line.

In Vitro Activation of rh-UCE by Furin

The N-terminal amino acid sequencing results of CHO- and insect-derived rh-UCE revealed that there are major structural differences between these two UCE samples. The CHO-derived UCE is not processed efficiently, i.e., ~65% pro-UCE and 35% mature UCE. In contrast, the insect-derived UCE is 100% mature UCE. The data suggests that the most of CHO-derived UCE must have either escaped the processing enzyme(s) that converts pro-UCE to the mature form or that the processing enzyme(s) responsible for this cleavage is defective in this CHO cell line. The human UCE contains a region that lies between pro-sequence and the start of the mature UCE sequence that may serve as a Furin cleavage site based on the primary amino acid sequence (unpublished data, S. Kornfeld & W. Canfield). Furin is a calcium-dependent serine protease that is endogenous to many mammalian cells. This protease requires a minimal furin cleavage site of Arg-X-X-Arg (SEQ ID NO:22).

The putative furin site in human UCE is NH_2-...RARARLPR ▲ DCT...-COOH

cleavage

(amino acids 42-52 of SEQ ID NO:18)

To determine whether furin is the enzyme responsible for the post-translational processing of pro-UCE to mature UCE the following experiment was performed.

A time-dependent analysis of UCE in the presence or absence of furin was performed. Twenty micrograms of CHO- and insect-derived UCE were incubated with 20 U furin at 30°C and 5μg of each UCE sample as removed after 0,6, 12, and 24 hours, respectively. Each sample was deglycosylated via PNGaseF and 200ng of each sample

subjected to SDS-PAGE followed by Western blotting using HPC4 mouse 1° antibody and horseradish-conjugated sheep-anti-mouse 2° antibody. All samples were also assayed for UCE activity and graphed as % increase in activity relative to the minus Furin samples.

Western blot analysis revealed that the CHO-derived UCE is sensitive to furin cleavage as shown by the progressive conversion of the pro-UCE to the mature form (Figure 1). The conversion of the pro-UCE to the mature UCE species is furin-dependent because the UCE sample that lacked furin remained as a mixture of pro- and mature UCE forms. In contrast, the insect-derived UCE is not cleaved by furin as shown by the single UCE form. The progressive conversion of the pro-UCE to the mature UCE species was confirmed by the increase in UCE activity (up to 130% increase in activity) relative to the minus furin sample (Figure 2). The insect-derived UCE did not show any increase in activity because it already exists as the mature form.

Defective Uncovering Enzyme in Furin-Deficient LoVo Cells

LoVo cells are derived from a human colon adenocarcinoma cell line that has shown to be void of furin activity (Lehmann et al (1996) Biochem. J. 317:803-809). The discovery that UCE requires furin for further processing above, prompted further investigation to determine the processing of UCE in furin deficient cells, and thus the UCE obtained.

LoVo cells were cultured to confluency and two lysosomal enzymes, β-glucuronidase (β-Gluc) and N-acetyl-β-D-glucosaminidase (NAcGluc) from the conditioned media was assayed for binding on a mannose-6-phosphate receptor column.

Sample **A** was conditioned medium from LoVo cells that was applied to the mannose-6-phosphate receptor column then eluted with 5 mM mannose-6-phosphate. The eluate was subsequently assayed for β-Gluc and NAcGluc activity. Sample **B** was conditioned medium from LoVo cells and dephosphorylated via alkaline phosphatase prior to mannose-6-phosphate receptor chromatography. Sample **C** was conditioned medium from LoVo cells that was treated with UCE in vitro prior to mannose-6-phosphate receptor chromatography. Sample **D** was conditioned medium from LoVo cells that was treated with UCE then alkaline phosphatase prior to mannose-6-phosphate receptor chromatography. The results of this study are shown below.

Obviously, numerous modifications and variations on the present invention are possible in light of the above teachings. It is therefore to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.